Antileukoprotease in Psoriatic Scales

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Antileukoprotease is known to be an antiproteolytic compound of mucous secretions in humans. While searching for peptide-like inhibitors of neutrophil-derived serine proteases in horny layers of human skin, we isolated a potent inhibitor of human leukocyte elastase (EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) from psoriatic scales. This inhibitor showed inhibitory constants for human leukocyte elastase of approximately $0.5-2 \times 10^{-10}$ M and for cathepsin G of $2-4 \times 10^{-9}$ N. The N-terminal amino acid sequence of the purified peptide matched the sequence of antileukoprotease and both peptides showed the same M_r on SDS-PAGE. Therefore, antileukoprotease may not only regulate serine protease activities in mucous secretions, but also in skin. Key words: antileukoprotease/human leukocyte elastase/cathepsin G. *J Invest Dermatol 101:305–309, 1993*

Antiproteolytic peptides and proteins are considered to be important regulators of tissue breakdown in inflammatory diseases that are caused by serine proteases of neutrophils. Human leukocyte elastase (HLE) and cathepsin G (CG) are thought to cause or augment the proteolytic tissue damage in lung emphysema [1] and adult respiratory distress syndrome (ARDS) [2]. Because earlier investigations revealed elevated activities of these enzymes in psoriasis [3,4], an inflammatory skin disease where neutrophils infiltrate the epidermis, detailed knowledge about inhibitors present in skin appears to be essential. Up to now, only three compounds with antiproteolytic activity have been demonstrated in skin: the serum-derived proteins α_1-proteinase inhibitor [5], α_2-macroglobulin [6], and elafin, an elastase-specific inhibitory peptide [7]. Because peptide-like inhibitors are thought to be much more efficient than antiproteolytic proteins in regulating proteolytic tissue breakdown [8], we looked for the presence of further antiproteolytic peptides in horny layers of human skin.

MATERIALS AND METHODS

The horny layers from approximately 100 psoriatic patients were pooled for extraction and purification to homogeneity of peptide-like inhibitors of human leukocyte elastase and cathepsin G.

Extraction Eighty grams of psoriatic scales were suspended in 100–200 ml distilled water, acidified to pH 2.8 by addition of 1% citrate and formic acid and diluted with 10% methanol (97%). After mechanical disruption by freeze-thawing (three times) as well as ultraturrax homogenization (10 min on ice) the solution was centrifuged (6000 × *g*, 10 min). The supernatants were concentrated by membrane ultrafiltration (Amicon YM5; Amicon Corp., Danvers, MA) and cleared by membrane filtration (5 μm). Buffer was changed to 0.01 M ammonium formate pH 4 by discontinuous membrane ultrafiltration (Amicon YM5) using three times the tenfold sample volume (diafiltration).

Purification The first purification step consisted of a cation exchange TSK CM 3-SW HPLC (150 × 7.5 mm; LKB, Bromma, Sweden). The extract (6 ml) was divided into two portions that were cromatographed separately. Proteins were eluted with a 30-ml linear gradient (flow, 1 ml/min) from 0.01 M–0.5 M ammonium formate at pH 4 and finally with 20 ml 0.5 M ammonium formate pH 3. Fractions of both runs showing inhibitory activity against HLE and CG were combined, concentrated, and diafiltered (Amicon YM5) against 0.1% trifluoroacetic acid (TFA).

Inhibitors were further purified by reverse-phase C_8 high-performance liquid chromatography (HPLC) (Nucleosil 300-7 C8, 250 mm × ¼ inch; Macherey-Nagel, Düren, Germany), elution buffer A 0.1% TFA in water, B 0.1% TFA in acetonitrile, eluting (flow, 3 ml/min) with 15 ml 20% B, followed by a 75-ml linear gradient of from 20% to 60% B and final elution with 100% B.

Relevant fractions were pooled, lyophilized, and further purified by reverse-phase C_{18}-HPLC (Nucleosil 300-7 C18, 250 mm × ¼ inch; Macherey-Nagel), elution buffer A 0.1% TFA in water, B 0.1% TFA in acetonitrile, eluting (flow, 2 ml/min) with 10 ml 20% B, followed by a 50-ml linear gradient of from 20% to 60% B and final elution with 100% B.

Relevant fractions were pooled and further purified on a cyanopropyl-column (250 × 4.6 mm, Baker, Phillipsburg, NJ), elution buffer A 0.1% TFA in water, buffer B 100% n-propanol, using a gradient from 10–35% buffer B over 25 min (flow, 1 ml/min) with subsequent elution with 100% buffer B.

The relevant fractions were finally purified on an analytical reverse-phase C_{18}-HPLC column (Nucleosil 100-5 C18, 250 × 4.6 mm; Bischoff, Leonberg, Germany), elution buffer A 0.1% TFA in water, buffer B 0.1% TFA in acetonitrile, eluting with a 30-ml linear gradient (flow, 1 ml/min) from 10% to 40% buffer B.

Inhibitory Activity Inhibition of HLE and CG was determined by the hydrolysis of specific tetrapeptide substrates according to Nakajima *et al* [9]. For HLE we used the substrate methoxysuccinyl-alanyl-alanyl-prolyl-valine-p-nitroanilide (Meo-Suc-Ala-Ala-Pro-Val-NA; Sigma, Deisenhofen, Germany), for CG the substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-NA; Sigma). Inhibitory activity was measured by 30-min preincubation of 20 ng of either enzyme (Elastin Products Corp., Pacific, MO) with 1–10 μl test sample in 200 μl 0.1 M HEPES, 0.5 M NaCl, 10% Me_2SO, pH 7.5 before adding 0.5 mM substrate in 800 μl of the same buffer. The absorbance at 405 nm was measured using an Eppendorf spectrophotometer.

For determination of K_i all solutions contained 1% bovine serum albumin (RIA grade; Sigma) and final substrate concentrations were 2 mM. HLE activity was active-site titrated with recombinant elafin. Titration with active-site–titrated HLE revealed the inhibitor to be more than 90% active. The *apparent* K_i was determined graphically according to the method of Green and Work [10] and the K_i was determined by a graphical method that compares data with simulated plots for tight binding inhibition with various K_i using Henderson's equation 13 [11]:

$$\frac{I_t}{1-\frac{v_i}{v_o}} = E_t + K_i\left(\frac{A_t + K_a}{K_a}\right)\frac{v_o}{v_i},$$

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Abbreviations: HLE, human leukocyte elastase; CG, cathepsin G; TFA, trifluoroacetic acid.

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with I_t, total concentration of inhibitor (0–60 nM); v_i, velocity in the presence of inhibitor; v_o, velocity without inhibitor; E_t, total concentration of enzyme (HLE 20 nM, CG 20 nM); K_i, dissociation constant for inhibitor; A_t, total concentration of substrate (2 mM); K_a, Michaelis constant for substrate (Meo-Suc-Ala-Ala-Pro-Val-NA, 0.14 mM; Suc-Ala-Ala-Pro-Phe-NA, 2.9 mM).

The equation was rearranged to

$$\frac{v_i}{v_o} = (K_a(E_t - I_t - K_i) - A_tK_i + \sqrt{A_t^2K_i^2 + 2A_tK_aK_i(E_t + I_t + K_i) + K_a^2[E_t^2 + 2E_t(K_i - I_t) + I_t^2 + 2I_tK_i + K_i^2]})/2K_aE_t,$$

to plot inhibition curves (v_i/v_o versus I_t by means of standard software (Sigma plot 4.1 or Microsoft Excel 4.0). I_t was varied [0,1, . . . ,60] nM assuming a 1 : 1 molar inhibition for HLE according to [12]. Inhibition curves for CG inhibition were based on the assumption of a 2 : 1 CG-inhibitor stoichiometry [12]. Therefore, the functional normality of the inhibitor I_t [0,2, . . . , 120] nN was used for determination of K_i.

Size-Exclusion HPLC For determination of the apparent molecular mass, samples were chromatographed on a TSK 2000 SW column (600 × 7.5 mm; LKB) equipped with a TSK SWP precolumn (75 × 7.5 mm) using 0.1% TFA at a flow of 1 ml/min. UV absorption was recorded at 215 nm and fractions were collected automatically (20 drops/fraction). M_r was calibrated with the peptides insulin B-chain fragment 22–36, glucagon, elafin (prepared according to [7]), aprotinin, ubiquitin, ribonuclease A, soybean trypsin inhibitor, ovomucoid trypsin inhibitor, and bovine serum albumin (calibrators obtained from Sigma or Serva, Heidelberg, Germany).

Sodiumdodecylsulfate-Polyacrylamide Gel-Electrophoresis (SDS-PAGE) The apparent molecular weight was determined by SDS-PAGE according to the method of Schägger and von Jagow [13] under reducing conditions (dithiothreitol, 100 mg/ml; Sigma) and as markers we used myoglobulin fragments MW-SDS-17 (Sigma) with molecular weights interpreted according to [14]. After fixation with 2% glutaraldehyde, polypeptides were stained using a silver staining kit (Sigma). For reference we used recombinant antileukoprotease kindly provided by Dr. Heinzel-Wieland, Grünenthal GmbH, Aachen, Germany.

Amino Acid Sequence Analysis The purified freeze-dried sample was solubilized in distilled water and an aliquot equivalent to 14 μg was subjected to N-terminal sequence analysis on an Applied Biosystems 477 pulsed liquid-phase sequencer with on-line analysis of the phenylthiohydantoins using a 120 A PTH analyzer. Gradients were used according to the recommendations of the manufacturer.

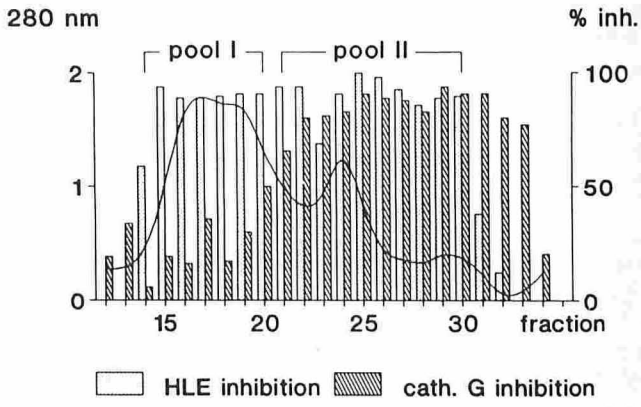

Figure 1. Elastase inhibitory activity following cation-exchange HPLC of an acidic extract of 40 g psoriatic scales. Absorbance at 280 nm is shown by the *solid line,* inhibitory activity of fractions against human leukocyte elastase (HLE) by *open bars,* and against cathepsin G (CG) by *hatched bars.*

RESULTS

To prove the presence of peptide-like HLE inhibitors in psoriatic skin, we extracted 80 g of psoriatic scales, collected from approximately 100 patients, and subjected the extract to cation-exchange HPLC in two portions. Figure 1 shows a representative experiment, which indicates that at least two inhibitory species could be separated by cation-exchange HPLC, one showing potent inhibitory activity against HLE (pool I) and a second showing inhibitory activity against HLE and CG (pool II). The relevant fractions (pool II) of both separations were combined and further purification was achieved by preparative reverse-phase C_8 HPLC (Fig 2*A*), which revealed a single peak of HLE and CG inhibitory activity. The inhibitory species was further purified on reverse-phase C_{18} HPLC (Fig 2*B*). Active fractions were pooled and chromatographed on a cyanopropyl-HPLC (Fig 2*C*). Final purification was achieved on an analytical reverse-phase C_{18} column (Fig 2*D*), which gave a single

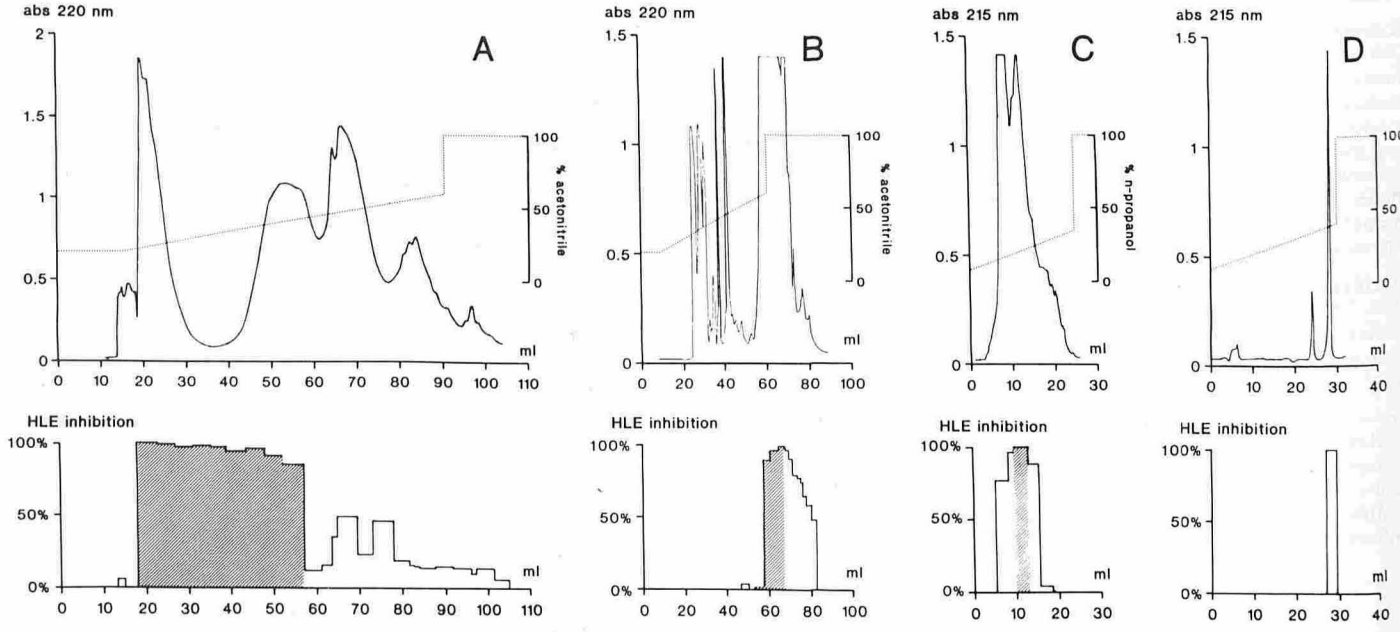

Figure 2. Purification of active fractions from cation-exchange HPLC (corresponding to pool II) by preparative RP C_8 HPLC *(A)*. Relevant fractions with HLE inhibitory activity *(hatched area)* were combined and further purified by preparative RP C_{18} HPLC *(B)*, cyanopropyl HPLC *(C)*, and RP C_{18} HPLC *(D)*. *Solid line,* absorbance at 220 or 215 nm; *dotted line,* gradient used.

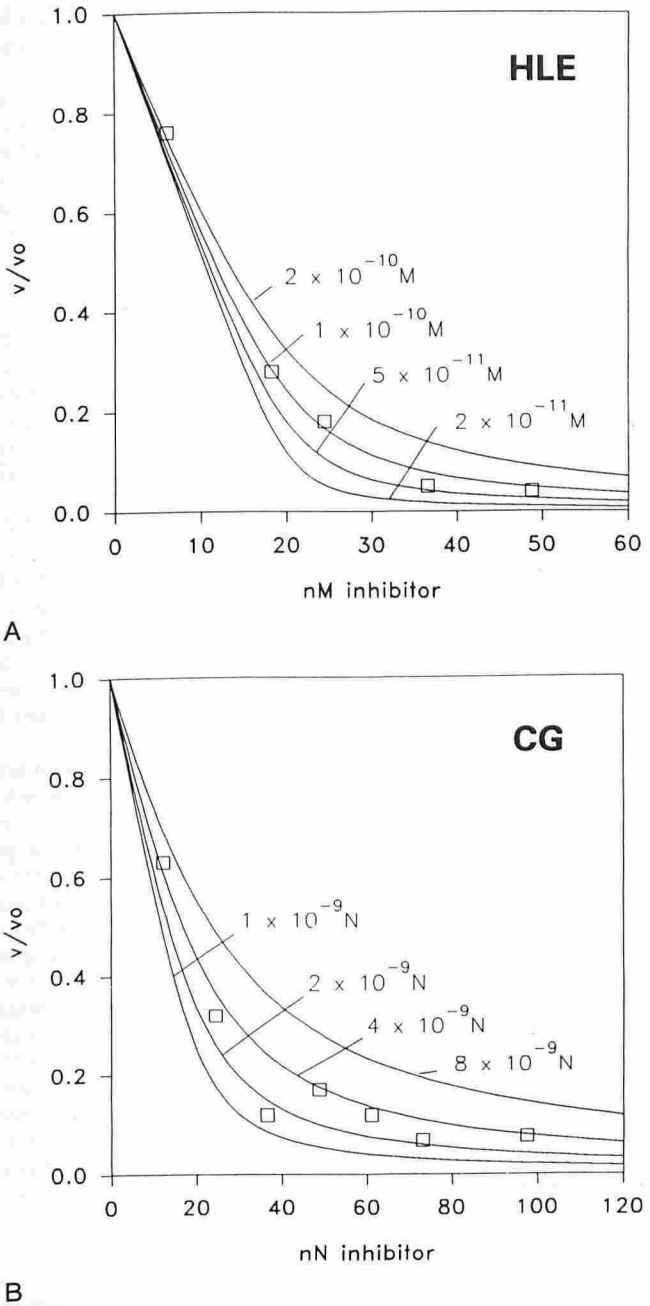

Figure 3. Inhibition of 20 nM human leukocyte elastase (HLE) and 20 nM cathepsin G (CG) by the purified inhibitor. *Solid lines,* simulated inhibition curves for different K_i according to equation 13 of Henderson [11] for reversible tight binding inhibitors. We assumed for HLE a 1 : 1 and for CG a 2 : 1 enzyme-inhibitor stoichiometry according to [12]. Therefore, K_i for HLE is given in molar concentration, the K_i for CG in functional normality.

symmetrical A215 peak with inhibitory activity against HLE plus CG (Fig 3).

A table of purification steps demonstrating protein content, inhibitory activity, and yield cannot be given, because at the beginning the extract consists of a mixture of enzymes and several inhibitors that make it impossible to evaluate the initial inhibitor concentration for each compound separately. Disregarding the presence of proteases, the total amount of extractable HLE inhibitory activity was 2.56 μmol per 80 g psoriatic scales. From this, 32.5 nmol antileukoprotease could be completely purified.

N-terminal amino acid sequence analysis of the first 37 residues revealed a partial sequence of antileukoprotease (Fig 4). Cysteine

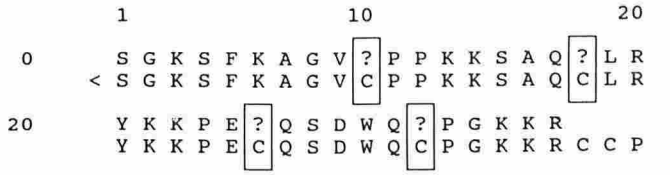

Figure 4. N-terminal amino acid analysis of elastase inhibitory peptide from psoriatic scales aligned with the sequence of antileukoprotease <, obtained from [15,17]. Cysteins have not been determined.

has not been determined, but all positions in which no residue could be detected match a cysteine in the amino acid sequence known for antileukoprotease. By TSK 2000 size-exclusion HPLC of the purified inhibitor, an apparent molecular mass of 13–14 kD could be demonstrated (Fig 5). The protein peak coeluted with the HLE-inhibitory activity (data not shown). In SDS-PAGE under reducing conditions (Fig. 6) the inhibitor showed a single band comigrating with recombinant antileukoprotease. Apparent molecular weight of antileukoprotease by SDS-PAGE was 14.7 kD, clearly overestimating the molecular mass of 11.7 kD of recombinant antileukoprotease that had been calculated from the amino acid sequence [15].

The apparent inhibition constant according to the method of Green and Work [10] proved to be 9×10^{-10} M for HLE and 3×10^{-9} M for CG consistent with the *apparent* K_i for HLE determined by Thompson *et al* [15]. K_i was determined using Henderson's equation 13 [11] for simulation of inhibition curves for a tight binding reversible inhibitor. K_a for the substrates (for HLE, Meo-Suc-Ala-Ala-Pro-Val-NA; for CG, Suc-Ala-Ala-Pro-Phe-NA) was taken from [9] and found to be identical under our experimental conditions. The K_i of antileukoprotease derived from psoriatic scales for HLE proved to be $0.5-2 \times 10^{-10}$ M, which is consistent with the K_i of 1.9×10^{-10} determined by Smith and Johnson [16]. For CG inhibition, we found the K_i to be about $2-4 \times 10^{-9}$ N, which is consistent with the K_i of 4×10^{-9} M known for antileukoprotease [16] and determined under the assumption of a 1 : 1 molar binding stoichiometry.

DISCUSSION

Antileukoprotease [17] is a very potent human serine protease, able to inhibit the neutrophil-derived serine proteases HLE and CG. This inhibitor is known to be present in a wide variety of human

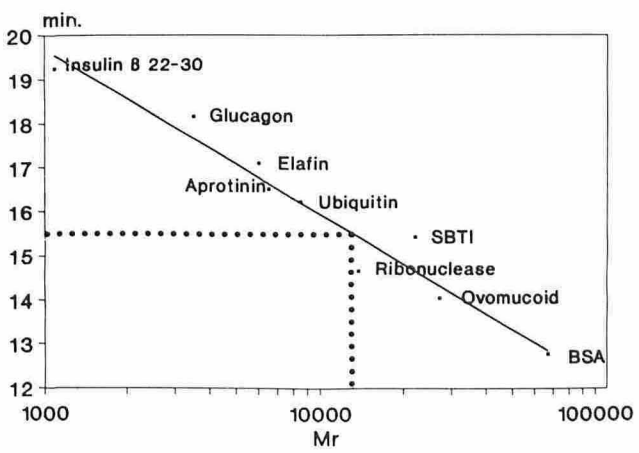

Figure 5. Determination of the apparent molecular weight by TSK 2000 SW size-exclusion HPLC. M_r was calibrated with the peptides insulin B-chain fragment 22–36 (MW 1,086), glucagon (3,483), elafin (5,999), aprotinin (6,512), ubiquitin (8,451), ribonuclease A (13,700), soybean trypsin inhibitor (22,000), ovomucoid trypsin inhibitor (27,000), and bovine serum albumin (67,000). *Dotted line,* elution time of antileukoprotease derived from psoriatic scales.

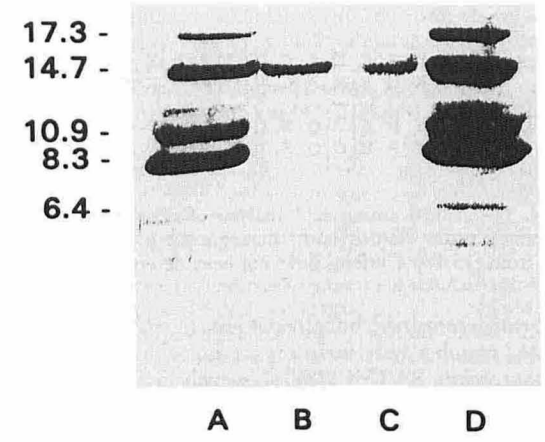

Figure 6. SDS-PAGE of antileukoprotease derived from psoriatic scales *(lane C)* in comparison with recombinant antileukoprotease *(lane B)*. As markers we used myoglobulin fragments *(lanes A,D)* with molecular weights given on the *left margin*. Polypeptides were stained by silver staining.

body fluids [18] such as bronchial mucus [19], parotid secretions [20], seminal fluid [21], and cervical mucus [22]. The concept of a protease-antiprotease imbalance, which is based on the assumption that tissue breakdown by excess HLE activity may be an important factor in the development of lung emphysema [23] and ARDS [24], has focussed attention on whether HLE inhibitors may regulate this process. The main HLE inhibitor of human serum, α_1-proteinase inhibitor, could be shown to be much less effective in preventing hydrolysis of tissue components than inhibitory active peptides like antileukoprotease or the medical leech-derived eglin C [8]. Therefore, antileukoprotease is thought to play a major role in the physiologic regulation of HLE activity in the lungs.

In human skin the concept of a protease-antiprotease imbalance has been proposed by Dubertret *et al* [25], who found marked epidermal proteolysis in psoriatic skin explants. At that stage the enzymatic activity could only be shown to be of serine protease type. There are three candidate serine proteases for the observed proteolytic activity: HLE, CG, and proteinase 3. In earlier investigations we showed that HLE activity (and CG activity; unpublished) is present on the surface of psoriatic lesions [4], but not on uninvolved psoriatic skin and in healthy controls. Because HLE [26], CG, and proteinase 3 are present in azurophilic granules of neutrophils, they may represent a suitable source of HLE and CG in psoriatic epidermis. Furthermore, HLE activity has been demonstrated to be present on the surface of diseased skin in contact dermatitis and atopic dermatitis [4], but the cellular origin of HLE activity in these skin diseases is not known.

There have been only a few investigations concerning HLE inhibitors in human skin. Fräki and Hopsu [27] found that α_1-proteinase inhibitor as well as α_2-macroglobulin, the HLE inhibitors of human serum, are present in human horny layers. Acid-stable peptide-like inhibitors of HLE have been demonstrated in psoriatic epidermis [28,29]. Purification and detailed characterization of these inhibitory activities revealed the presence of an elastase-specific inhibitor, elafin [7], which has also recently been purified from bronchial mucus [30].

A second HLE inhibitor can be extracted from psoriatic scale material. We have developed a purification procedure using sequential HPLC to purify this inhibitor to homogeneity. The *apparent* K_i of the purified inhibitor for HLE and CG inhibition were comparable to those reported for antileukoprotease [16] derived from bronchial mucus. Amino acid sequence analysis (Fig 4) revealed the N-terminal sequence of antileukoprotease [15,17], and the apparent molecular masses in SDS-PAGE of antileukoprotease derived from psoriatic scales and of recombinant human antileukoprotease were identical (Fig 6). Therefore, the second HLE inhibitor derived from psoriatic scales seems to be identical with antileukoprotease from mucous secretions in terms of N-terminal amino acid sequence, molecular size, and inhibitory properties.

Approximately 400 nmol antileukoprotease/kg psoriatic scales can be completely purified from psoriatic scale material. Therefore, the concentration in the fluid phase of psoriatic scales is at least 400 nM. The fact that the concentration of antileukoprotease exceeds the K_i of 200–400 pM for HLE at least 1000 times indicates that antileukoprotease is present in psoriatic scales in physiologically relevant concentrations.

The cellular origin of antileukoprotease in skin remains to be established. In human serum antileukoprotease serum level is 126 μg/ml [31], which is about 10 nM. Therefore, it seems unlikely that antileukoprotease is simply derived from serum contamination. Specific accumulation of serum-derived antileukoprotease seems to be possible. This is supported by the findings of Gast *et al* [32], who found approximately 15% of radiolabeled antileukoprotease 5 min after being administered intravenously to rats in the skin. Another possible source that can be considered may be sweat glands.

The presence of two potent peptide-like inhibitors of HLE in psoriatic scale material, antileukoprotease inhibiting HLE plus CG, as well as elafin-inhibiting HLE plus proteinase 3 [33], supports the hypothesis that at least in psoriasis the epidermis is protected against proteolytic degradation. Localized excess of HLE, e.g., in the intercellular space, however, might cause a protease-antiprotease imbalance, leading to degradation of extracellular matrix components followed by the formation of spongiosis, bullae, blisters, or pustules, as seen in a variety of inflammatory skin disorders.

Therefore, not only ARDS and lung emphysema, but also acute and chronic inflammatory skin disorders may profit from the development of serine protease inhibitors for use in therapy. Some evidence that reconstitution of the HLE-HLE inhibitor balance in psoriasis might be of therapeutic value is given by the fact that bathing in hypertonic salt solutions removes HLE from the surface of psoriatic lesions [3,34]. Furthermore, it could be shown that tannin, which is used as an adjuvant in topical therapy of vesiculating skin disorders, is a potent irreversible inactivator of HLE, but not of other closely related serine proteases [35]. In addition, preliminary data show that topical application of α_1-proteinase inhibitor exerts beneficial therapeutic effects in the therapy of atopic dermatitis [36].

In conclusion, one of the most potent serine protease inhibitors in humans, antileukoprotease, is present in psoriatic scales in concentrations that are likely to be sufficient for inhibition of the neutrophil-derived serine proteases HLE and CG. The cellular origin of antileukoprotease in skin and the localization at the cellular level remain to be investigated.

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ANNOUNCEMENT

A symposium entitled "Melanin: Its Role in Human Photoprotection" will be held at the Hyatt Regency Crystal City, Washington, D.C. National Airport, U.S.A., March 11–12, 1994. The objective of the symposium is to review for the scientific, regulatory, business communities and the general public at large the current knowledge of the role of melanin in UV protection. The organizing committee is chaired by Dr. Miles R. Chedekel. Dr. Ago B. Ahene is the secretary. The registration desk will open at 3:00 p.m. on Thursday, March 10, 1994, and will be followed in the evening by a welcoming reception at the Hyatt Regency. The scientific sessions will begin at 9:00 a.m. on Friday, March 11, 1994. The conference will end on Saturday, March 12, 1994.

The tentative program is divided into five plenary sessions and will also include several Workshops and Round-table Discussions. The topics include chemistry and physics of melanins, melanin metabolism, natural distribution of melanins and photoprotection, absorptive and light-scattering properties of melanins and its precursors, melanin and free-radicals, and round-table discussions.

A second announcement and call for papers will be circulated this summer. For further information, please contact the Symposium Secretariat, Dr. Ago B. Ahene, Melanin Symposium Secretariat, 3696 Haven Avenue, Redwood City, California, 94063, U.S.A. Telephone, (415) 366-2626; Facsimile, (415) 368-4470.